Identification of a plastid protein involved in vesicle fusion and/or membrane protein translocation

(protein insertion machinery)

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ABSTRACT Structural evidence has accumulated suggesting that fusion and/or translocation factors are involved in plastid membrane biogenesis. To test this hypothesis, we have developed an in vitro system in which the extent of fusion and/or translocation is monitored by the conversion of the xanthophyll epoxide (antheraxanthin) into the red ketocarotenoid (capsanthin). Only chromoplast membrane vesicles from red pepper fruits (Capsicum annuum) contain the required enzyme. Vesicles prepared from the mutant yellow cultivar are devoid of this enzyme and accumulate antheraxanthin. The fusion and/or translocation activity is characterized by complementation due to the synthesis of capsanthin and the parallel decrease of antheraxanthin when the two types of vesicles are incubated together in the presence of plastid stroma. We show that the extent of conversion is dependent upon an ATP-requiring protein that is sensitive to N-ethylmaleimide. Further purification and immunological analysis have revealed that the active factor, designated plastid fusion and/or translocation factor (Pftf), resides in a protein of 72 kDa. cDNA cloning revealed that mature Pftf has significant homology to yeast and animal (NSF) or bacterial (Fsh) proteins involved in vesicle fusion or membrane protein translocation.

The capacity of a plastid to house several biosynthetic pathways or perform photosynthesis is strictly linked to its state of differentiation. During differentiation, membranes are modified by new sets of proteins targeted and inserted into specific sites. Vesicles derived from the inner plastid membranes may participate in thylakoid formation by fission and fusion to membrane acceptors on new thylakoids (1–3). This is reminiscent of vesicle fusion in animals and yeast (4–6), which involves a cytoplasmic protein, known as NSF [for N-ethylmaleimide (NEM)-sensitive factor] which facilitates this process. In plants, we are unaware of component proteins involved in this process (7–9). A major problem for this type of study is that in developing chloroplasts the sequential steps of vesicle budding and fusion are very transient. Thus this phenomenon is usually observed when the normal pathway of chloroplast differentiation is halted—for example, when the greening period is extended by different light regimes (10) or when plants are placed under various stress conditions, including cold treatment (11) or anoxia (12). On the other hand, chloroplasts, which naturally develop in sink tissue, in the absence of selective pressure for thylakoid differentiation and subsequent photosynthetic electron transfer, display several facets of vesicle budding and fusion as judged indirectly by electron microscopy (13). Therefore, this organelle could represent a model system for isolating component proteins involved in vesicle fusion and/or membrane protein translocation.

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FIG. 1. Electron micrographs showing the formation and the evolution of the plastid membrane vesicles in C. annuum plastids. The vesiculation of the inner chloroplast envelope membrane shown by arrows (A) is followed, during the chloroplast-to-chromoplast transition stages (B), by increased detachment and accumulation of the membrane vesicles in the plastid stroma, where putative vesicle fusion is indicated by arrowheads. (Bar = 0.5 μm.)

Using isolated Capsicum chromoplasts, we have purified and characterized a plant factor catalyzing the ATP-dependent fusion and/or translocation of an integral protein of chloroplast vesicle donors to chromoplast vesicle acceptors. Cloning and sequencing of this protein, designated Pftf (for plastid fusion and/or translocation factor), reveal similarities to animal (5) and yeast (6) NSF's and bacterial Fsh (14–16).

MATERIALS AND METHODS

Preparation of Chromoplast Membrane Vesicles. The membrane vesicles were purified from intact chromoplasts of red

Abbreviations: NEM, N-ethylmaleimide; NSF, NEM-sensitive factor.
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‡The sequence reported in this paper has been deposited in the GenBank database (accession nos. X80755).
pepper fruits (*Capsicum annuum* L. cv. Yolo Wonder) or yellow pepper fruits (*Capsicum annuum* L. cv. Jaune de Pignerolle) (17) as described (18).

**In Vitro Assay of Pftf Activity.** Chromoplasts from red pepper fruits contain the carotenogenic enzyme capsanthin synthase (a ketoxanthophyll synthase) (18), which catalyzes the conversion of antheraxanthin into capsanthin. Vesicles prepared from the mutant yellow cultivar are devoid of this enzyme and accumulate xanthophyll epoxides such as antheraxanthin. We developed an *in vitro* assay wherein fusion is characterized by complementation due to the synthesis of capsanthin and the parallel decrease of antheraxanthin. Reaction components (final reaction volume, 250 μl) included chromoplast vesicles containing 500 μg of total protein each from the red and yellow mutants, 200 μg of stromal proteins (due to the putative involvement of additional factors), or purified Pftf in 50 mM Tris-HCl, pH 7.6/5 mM ATP/10 mM MgCl₂. The yellow vesicles also contained 25 μg of antheraxanthin at the start of the assay. In separate treatments plastid stromal proteins were heated at 90°C or treated with 10 mM NEM at 4°C for 30 min and then passed through a PD-10 gel filtration column (Pharmacia) before addition to the reaction mixture. During the isolation and purification of Pftf, the activity of each fraction was determined with the same incubation mixture except that the stromal proteins were routinely treated with 10 mM NEM as described above. Reaction mixtures were incubated at 25°C for 60–120 min, 750 μl of chloroform/methanol (2:1, vol/vol) was added to stop the reaction, and centrifuged. HPLC analysis measured the conversion of antheraxanthin into capsanthin (18).

**Purification of Pftf from Chromoplast Stroma.** Stromal proteins (750 mg) were prepared from *C. annuum* fruitoplasts (17) by subjecting the intact plastids to osmotic shock in 50 mM Tris-HCl, pH 7.6/5 mM dithiothreitol/0.1 mM ATP, followed by ultracentrifugation (100,000 × g for 60 min). The supernatant was adsorbed onto fast-flow Q-Sepharose (Pharmacia) and proteins were eluted with a linear 50–300 mM NaCl gradient in 50 mM Tris-HCl, pH 7.6. The active fractions, which were eluted at 0.15–0.2 M NaCl, were pooled and further fractionated in Mono Q (HRS/5 column; Pharmacia) with the same gradient. The active fractions were supplemented with 5 mM MgCl₂ and finally loaded onto an ATP-agarose (Sigma) column (1 cm × 2 cm). After the column was washed with 50 mM Tris-HCl, pH 7.6/5 mM MgCl₂/0.1 M NaCl, Pftf was eluted with the same buffer supplemented with 5 mM ATP. The active fractions were pooled, concentrated against dry sucrose, and subjected to preparative SDS/PAGE to purify Pftf for raising polyclonal antiserum in rabbits (19).

**Other Procedures.** SDS/PAGE and immunoblotting were carried out as described (19). Histochemical localization of Pftf by immunofluorescence was as described (17, 21). cDNA cloning from a pepper library (20, 21) and all standard molecular biology techniques were as described (22). For regular electron microscopy, pericarp tissues and isolated membrane vesicles were fixed and processed as shown previously (23).

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**Table 1. Purification of the Pftf from *C. annuum* chromoplasts**

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein, mg</th>
<th>Normalized specific activity</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastid stroma</td>
<td>750</td>
<td>0.8</td>
<td>1</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>150</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Mono Q HRS/5</td>
<td>30</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>ATP-agarose</td>
<td>1</td>
<td>100</td>
<td>125</td>
</tr>
<tr>
<td>Preparative SDS/PAGE</td>
<td>0.3</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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<FIG. 2. In vitro fusion between chromoplast vesicle purified from yellow mutant and normal red *C. annuum* fruits is controlled by a plastid NEM-sensitive factor, Pftf. Fusion was monitored by the conversion of antheraxanthin into capsanthin. The reaction conditions tested included the absence of stromal proteins (bar 1), the presence of ATP alone (bar 2), the presence of ATP and stromal proteins (complete reaction mixture) (bar 3), and the reaction mixture 3 depleted of ATP (bar 4). In addition, the effect of heating the stromal proteins at 90°C (bar 5) or treating the stromal proteins with 10 mM NEM before incubation (bar 6) was investigated. Each bar corresponds to the mean ± SD of three experiments.>

<FIG. 3. SDS/PAGE and immunoblot analysis of purified Pftf. Lane 1, stromal extract used as starting material; lane 2, active fraction after Mono Q column chromatography; lane 3, followed by ATP-agarose column chromatography; lane 4, immunoblot analysis of total stromal proteins. Molecular mass markers are indicated at left.>

<FIG. 4. Cellular immunolocalization of Pftf. Preimmune serum gives no signal (4) and immune serum indicates positive labeling of plastids (B).>
RESULTS AND DISCUSSION

Ultrastructural Evidence for Plastid Vesicle Budding and Fusion in Vivo. At the onset of chloroplast-to-chromoplast differentiation an extensive vesiculation of the inner plastid envelope membrane followed by detachment and fusion to give achlorophyllous membranes takes place (Fig. 1). It has been hypothesized that vesicles pinching off from the plastid envelope could represent a mechanism for the transport of low

Fig. 5. Structure of C. annuum Pftf cDNA and the predicted amino acid sequence. Numbering is from the 5' end of the DNA sequence.
molecular weight compounds and macromolecules involved in intraplastid membrane biogenesis (1, 2). Though direct demonstration of this phenomenon is still lacking, biochemical arguments partly sustaining this hypothesis could be inferred from the fact that galactolipids synthesized in the plastid envelope must be transported to the thylakoid membranes (24). This leaves open the possibility that specific fusion or translocation factors may reside in plastids.

In Vitro Test for Vesicle Fusion or Protein Translocation. Purified chromoplast membrane vesicles from yellow and red types were combined in vitro, and we followed the conversion of antheraxanthin into capsanthin. This was measured by the disappearance of antheraxanthin and the concomitant increase in capsanthin content, estimated to represent 2.5% conversion after 120 min (Fig. 2). Addition of 5 mM ATP to the mixture doubled the capsanthin content. Addition of stromal extracts from either red or yellow fruit chromoplasts to the incubation mixture in the presence of ATP resulted in 20% conversion of antheraxanthin into capsanthin (Fig. 2). The presence of ATP was strictly required, since its depletion from the incubation medium resulted in only 7% conversion (Fig. 2). The stimulating factor was completely inhibited by heating the stromal extract at 90°C for 10 min prior to incubation, suggesting that the stromal factor was proteinaceous (Fig. 2). Pretreatment of the stromal extract alone with 10 mM NEM also resulted in strong inhibition of capsanthin formation (Fig. 2). Collectively, these data suggested that a rearrangement of the membrane system occurred in order to allow the transformation of antheraxanthin into capsanthin and that a proteinaceous vesicular fusion and/or translocation factor similar to that described in other organisms (5) resides in the plastid stroma. This protein factor was termed Ptf, for plastid fusion and/or translocation factor.

Characterization of Ptf. The stromal proteins were subsequently fractionated on Q-Sepharose, Mono Q, and ATP-agarose columns (see Materials and Methods). SDS/PAGE of the active fractions eluted from ATP-agarose revealed a main band at 72 kDa and a minor band at 32 kDa (Fig. 3, lane 3).

When the most active fractions were subjected to gel filtration on Sephacryl S-200, only the 72-kDa protein was catalytically active. Therefore, the ATP-agarose fractions were pooled and further subjected to preparative SDS/PAGE. Treatment of the ATP-agarose-purified protein factor with 10 mM NEM reduced the activity by 80%. Antibodies raised against the 72-kDa protein produced a single band after immunoblot analysis of the total stromal proteins (Fig. 3, lane 4). Furthermore, incubation of the chromoplast stroma with increasing concentrations of antibodies, followed by ultracentrifugation before assay, induced a concentration-dependent reduction of the activity. A summary of the purification is shown in Table 1. Immunocytochemical analysis was performed with the antibodies directed against the 72-kDa protein and pericarp disk of red C. annuum fruits. The preimmune serum gave no labeling (Fig. 4A), whereas the immunofluorescence staining pattern obtained with the immune serum corresponded to the distribution of the plastids in the pericarp section. Therefore the 72-kDa protein is localized in the plastids (Fig. 4B). The molecular mass of Ptf, 72 kDa, corresponds to that of the NSF from Chinese hamster (25).

cDNA Cloning and Expression of Ptf. A full-length Ptf cDNA from a λgt11 pepper fruit library was isolated by using the antibodies against the 72-kDa protein to screen the library (20–22). The sequence of the full-length clone (Fig. 5) encodes a protein of 76,900 Da that most likely is the precursor of Ptf. Indeed, the amino-terminal end of the precursor has several features of plastid transit peptides, including the presence of few acidic residues and several serine residues (26). In addition, the putative Ptf transit peptide starts with the sequence Met-Ala-Thr and has the putative cleavage site Val-Lys-Ala-Ser-Leu, which are identical, respectively, to those of rubisco activase (27) and platocyanin (28) transit peptides. Under these conditions the deduced molecular mass of the mature peptide is 71 kDa, a value very similar to the 72 kDa obtained after SDS/PAGE. The mature protein sequence shares significant homology (Fig. 6) with the proteins NSF (51% similarity and 28% identity; ref. 4), Sec18p (53% similarity and
27% identity; ref. 6), and FtsH (73% similarity and 56% identity; ref. 14) and the bacterial division protein Bs (75% similarity and 60% identity; GenBank/EMBL accession no. D26185). Further analysis of the deduced peptide sequence reveals that Pftf, in contrast to NSF, shows only one typical ATP-binding domain with the two characteristic motifs A and B (Fig. 6) (29–31), which are probably involved in the ATPase function of Pftf. Available data concerning the protein NSF indicate that the sensitivity to NEM is due to the alklylation of the cysteine residue present in motif A (31). In Pftf the putative cysteine residue in motif A is replaced by threonine, but in motif B a conserved cysteine is present in FtsH as well as in Bs (Fig. 6). This conserved cysteine residue could therefore explain the NEM sensitivity of Pftf activity. This possibility is strengthened by the fact that the protein p97, which displays strong homology to the protein NSF, the sensitivity to NEM is also due to the presence of a cysteine residue only in the second ATP-binding domain (32). At the other extreme, Sec18p has also a strong homology to NSF but has less sensitivity to NEM, since no cysteine residue is present in the two ATP-binding domains (6).

Northern blot analyses of Pftf mRNAs, normalized to the same ribosomal RNA, showed that expression of the gene was constitutive in leaves and developing fruits of pepper (data not shown).

Potential Involvement of Pftf in Membrane Biogenesis. First, our data strengthen the putative role of the inner plastid envelope in the formation of thylakoid membrane or inner plastid membrane components (1–3, 13, 33). Indeed, this phenomenon can be traced from photosynthetic procaroytes to higher plants (10). In bacteria, the vesiculation of the periplasmic membrane to give photosynthetic membrane is one of the most primitive aspects of this phenomenon. In cyanobacteria, the progenitors of higher plant plastids, the galactolipid composition of the periplasmic membrane and the thylakoids argues for this tight cooperation (34). This cooperation could be mediated by mass flow of material through the inner plastid envelope membrane vesicles or by detachment of the envelope vesicles and fusion to the thylakoid membrane target. Second, our results suggest that Pftf could be involved in the insertion of integral protein into plastid membranes. As such Pftf is a likely candidate for the assisted insertion of the light-harvesting chloroplast protein into chloroplast thylakoids, since this process requires a soluble stromal factor sensitive to NEM (35, 36).

Finally, the role of Pftf in plastid biogenesis is further strengthened by the fact that sequence analysis of the plastid genome of red alga revealed the presence of an open reading frame homologous to NSF and other bacterial proteins involved in protein translocation (37–39). The identification of a Pftf provides an opportunity to further study this class of proteins involved in the assembly of plastid membranes.

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